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(54) Title: METHOD FOR MEASURING PLK4 ACTIVITY

(57) Abstract: The present invention describes a method for determining the activity of PLK4 comprising the steps of: providing a sample to be tested for PLK4 activity, determining the level of autophosphorylation of S305 of PLK4 in said sample, and determining the activity of PLK4 activity in the sample of cells, wherein the level of S305 autophosphorylation directly correlates with the level of PLK4 activity.

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METHOD FOR MEASURING PLK4 ACTIVITY

FIELD OF THE INVENTION

- 5 The present invention relates to a novel method for measuring PLK4 activity and/or PLK4 activation status, especially in cells and tissue samples.

BACKGROUND OF THE INVENTION

- 10 Polo-like kinases (PLKs) are key enzymes that control mitotic entry of proliferating cells and regulate many aspects of mitosis necessary for successful cytokinesis, including centrosome duplication and maturation; DNA damage checkpoint activation; bipolar spindle formation; Golgi fragmentation and assembly; and chromosome segregation (Barr, F. A. et al., Nat. Rev. Mol. Cell Biol. 2004, 5, 429-441). PLKs are found in organisms as diverse as yeast and human and contain two conserved domains,
- 15 the N-terminal catalytic kinase domain and a C-terminal region composed of the so-called polo-boxes. In yeasts a single PLK exist, whereas four distinct PLKs have been identified to date in mammals. Whereas PLK1, PLK2 and PLK3 are expressed in all tissues and structurally homologous in that they comprise the N-terminal catalytic kinase domain and two polo-boxes, PLK 4 differs not only in structure, compared to
- 20 the other PLKs it has only one polo-box, but also in the distribution of PLK4 mRNA in adults that is restricted to certain tissues such as testes and thymus (Karn, T. et al., Oncol. Rep. 1997, 4, 505-510; Fode, C. et al., Proc. Natl. Acad. Sci. USA 1994, 91, 6388-6392). It is still under investigation whether these differences also result in a unique physiological role for PLK4.
- 25 Given the established role of PLKs as mitotic regulators, they have been regarded as validated mitotic cancer targets for a number of years. In addition, recent studies demonstrate that changes of intracellular levels of PLKs are involved in the control of cell growth. For example, PLK1 when fused to an antennapedia peptide and efficiently internalized into cells caused an inhibition of cancer cell proliferation (Yuan, J., et al.,
- 30 Cancer Res. 62, 2002, 4186-4190), whereas downregulation of PLK1 by antisense induced the growth inhibition of cancer cells (Spankuch-Schmitt, B., et al., Oncogene 21, 2002, 3162-3171). PLK2 was recently found to be a novel p53 target gene and RNAi silencing of PLK2 leads to mitotic catastrophe in taxol-exposed cells (Burns,

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TF., et al., Mol Cell Biol. 23, 2003, 5556-5571). For PLK3 it was found that it induces cell cycle arrest and apoptosis through perturbation of microtubule integrity (Wang, Q., et al., Mol Cell Biol. 22, 2002, 3450-3459) and PLK4 was shown to be transcriptionally repressed by p53 and induces apoptosis upon RNAi silencing (Li, J., et al., Neoplasia 7, 5 2005, 312-323). PLK4 was also found to be required for centriole duplication and flagella development. The absence of centrioles, and hence basal bodies, compromises the meiotic divisions and the formation of sperm axonemes (Bettencourt-Dias M., et al., Current Biology 15, 2005, 2199-2207).

All of this confirms that targeting PLKs with conventional small-molecule agents may 10 be a valid and effective anticancer strategy with potential to synergize with established DNA-damage and antimitotic chemotherapies.

Relatively few reports of selective small-molecule PLK inhibitors have appeared to date.

15 Although multiple gene products have been identified to be crucial for PLK4 controlled centriole biogenesis, a physiological relevant substrate of PLK4 is yet to be described (Kleylein-Sohn, J., J. Westendorf, M. Le Clech, R. Habedanck, Y.D. Stierhof, and E.A. Nigg. 2007. Plk4-induced centriole biogenesis in human cells. *Dev Cell*. 13:190-202). Therefore, it is currently not possible to measure PLK4 activity easily.

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AIMS OF THE INVENTION

A primary aim of the present invention comprises providing an easy and reliable method to measure PLK4 activity and/or PLK4 activation status, especially in cells and tissue samples.

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SUMMARY OF THE INVENTION

The present invention provides a method for determining the activity of PLK4 comprising the steps of:

- (a) providing a sample to be tested for PLK4 activity,
- 30 (b) determining the level of autophosphorylation of S305 of PLK4 in said sample, and

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- (c) determining the activity of PLK4 activity in the sample of cells, wherein the level of S305 autophosphorylation directly correlates with the level of PLK4 activity.

In one embodiment of the method of the invention, the activity of PLK4 is determined using an immunostaining procedure with an antibody reagent that is specific to the S305 autophosphorylation site on PLK4. In one embodiment the immunostaining procedure is immunofluorescent detection of autophosphorylated PLK4, using for example cultured cells in a flask or plate, or cell smears from tissue samples, biopsies or needle aspirates. In another embodiment the immunostaining procedure is immunohistochemical detection of autophosphorylated PLK4, using for example cell smears from tissue samples, biopsies or needle aspirates, or tissue sections that have been fixed to preserve the tissue structure, e.g. by freezing, or by paraformaldehyde fixation and paraffin embedding. Standard methods for cell or tissue fixation, binding of antibody reagents, and labeling or staining can be employed in these immunostaining procedures (e.g. see Using Antibodies, A Laboratory Manual, edited by Harlow, E. and Lane, D., 1999, Cold Spring Harbor Laboratory Press (e.g. ISBN 0-87969-544-7), particularly chapters 5 and 6 on staining cells and tissues).

The step of determining the level of autophosphorylation of S305 of PLK4 in said sample can be performed for example by using a phosphorylated S305-specific antibody, preferably fluorescently or radioactively labeled, or for example by using a sandwich ELISA assay in which a first antibody reagent is specific to PLK4 protein and a second antibody reagent is specific to the S305 autophosphorylation site on PLK4, or by electrophoretic separation of the proteins in the sample and immunoblot analysis using an antibody reagent specific to the S305 autophosphorylation site on PLK4. Alternatively, S305 antibodies can be used for the measurement of PLK4 phosphorylation level in circulating blood cells such as Leukocytes or circulating tumor cells as a surrogate marker for the activity inhibitors aimed at reducing PLK4 activity. Fixation and permeabilization of circulating blood cells and analysis using state of the art methods such as Fluorescence Activated Cell sorting (FACS) or derived methods are included.

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In one embodiment of the method of the invention, the level of autophosphorylation of S305 of PLK4 is determined using a sandwich ELISA assay in which a first antibody reagent is specific to PLK4 protein and a second antibody reagent is specific to the S305 autophosphorylation sites on PLK4. In a preferred embodiment of these methods

5 the first antibody reagent is adsorbed onto a surface (e.g. a plate or dish, e.g. a 96-well plate), a cell extract is prepared from the sample of cells to be tested such that the PLK4 protein is solubilized (e.g. using a protein solubilizing agent such as SDS or PROTEOEXTRACT®), the cell extract is treated if necessary to ensure that any agents used to solubilize PLK4 will not affect antibody binding to PLK4 (e.g. by dilution,

10 addition of detergents such as Triton X-100), PLK4 protein in the extract is adsorbed onto the surface by contacting with the first antibody, and the phosphorylation level of the adsorbed PLK4 is quantitated by contacting with a labeled second antibody reagent that is specific to the S305 autophosphorylation sites on PLK4. Due to their speed and simplicity, such ELISA methods are particularly advantageous where a rapid assay of

15 PLK4 activity is required, or where large numbers of sample have to be analyzed, e.g. in a high- throughput compound screen. ELISA methods are well known to those of skill in the art, e.g. see International Patent Publication No. WO 95/14930, or Using Antibodies, A Laboratory Manual, edited by Harlow, E. and Lane, D., 1999, Cold Spring Harbor Laboratory Press, (e.g. ISBN 0- 87969-544-7).

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In alternate embodiments of the present invention other standard immunoassay formats may be used in place of the sandwich ELISA assay format for the determination of the level of autophosphorylated PLK4, e.g. an antigen competition assay with phosphorylated PLK4 adsorbed onto a solid phase (e.g. a 96- well plate), with the

25 amount of phosphorylated PLK4 in the sample being quantitated by its competition with the solid phase bound PLK4 for binding to a labeled S305-autophosphorylation-site-specific antibody in solution.

In another embodiment, a dot blot assay may be used for the determination of the level

30 of autophosphorylated PLK4. Accordingly, the latter embodiment provides a method for determining the activity of PLK4 comprising the step of adsorbing the PLK4 protein onto a membrane (e.g. a hydrophobic membrane, nitrocellulose, nylon), and

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contacting with a labeled antibody reagent that is specific to the S305 autophosphorylation site on PLK4.

In another embodiment of the method of the invention, the level of phosphorylation of
5 PLK4 is determined by electrophoretic separation of the proteins in the sample and immunoblot analysis using an antibody reagent specific to the S305 autophosphorylation site on PLK4. In a preferred embodiment of the above methods, electrophoretic separation of the proteins in the sample is achieved by SDS- PAGE.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 describes the identification of PLK4 autophosphorylation sites within the N-terminus of the kinase:

(A) A diagram showing the position of the kinase domain (KD), crypto Polobox (CPB), Polobox (PB) and PEST sequences (numbered 1, 2 and 3) within
15 PLK4. Presented in the box is an autoradiogram showing the results of an in vitro kinase assay using full length PLK4.

(B) The results of in vitro kinase assays using truncated PLK4 1-597 fused to SUMO. Samples of the purified protein were separated on gels and subjected to western blotting (WB) using an anti-PLK4 antibody,
20 coomassie blue (CB) staining and autoradiography (AR).

(C) An autoradiogram of in vitro kinase reactions carried out using different truncations of PLK4 and a mutant version with methione substituted for lysine at position 41 (K41M) to render the kinase inactive.

Figure 2 shows multiple autophosphorylation sites exist within PLK4:

25 (A) A diagram showing the PLK4 fragments that were fused to SUMO, expressed and purified from bacteria.

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- (B) SUMO-PLK4 fragments were used as substrates in in vitro kinase assays using SUMO-PLK4 1-285. Autoradiography demonstrated that all of the fragments were phosphorylated to varying extent with the exception of fragment 888-971 where no phosphorylation could be detected. It should be noted that fragment 365-639 migrated at a similar rate as SUMO-PLK4 1-285, resulting in the masking of the phosphorylation from this fragment as the N-terminus of PLK4 readily autophosphorylates. Sypro Ruby staining of the gel was carried out to verify the quantities of SUMO-PLK4 fragments loaded.
- 10 Figure 3 describes the identification of autophosphorylation sites in PLK4 by in silico and peptide array screening.
1. Potential autophosphorylation sites were identified using a program that searched for a consensus phosphorylation sequence in PLK4 which had been derived after the screening of multiple peptide libraries.
 - 15 2. A custom made peptide library was phosphorylated with PLK4 1-285 to validate the phosphorylation sites identified in silico. Presented are autoradiograms of the gels loaded with peptides that were found to be significantly phosphorylated by PLK4 (marked with asterisks). The peptide numbers are noted on the tops of the gels and sequences are given in the
20 boxes with potential phosphorylation sites marked with squares and S/T to A mutations marked with circles. Serine residue 305 appeared to be the major autophosphorylation site within PLK4. The plus sign indicates a positive control carried out using a 12 amino acid peptide from RAF containing serine 338 closely matching the consensus phosphorylation
25 sequence of PLK4.

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Figure 4 shows that serine 305 of PLK4 is autophosphorylated in vitro and in vivo.

- (A) Samples of in vitro phosphorylated PLK4 1-367, PLK4 1-367 K41M and lambda phosphatase treated PLK4 1-367 were analysed by Sypro Ruby staining, autoradiography and western blotting with anti-PLK4 pS305 antibody. The anti-PLK4 pS305 antibody did not recognize PLK4 1-367 after lambda phosphatase treatment or the inactive form of the kinase, however, strong autophosphorylation of PLK4 1-367 was observed.
- (B) Lysates were prepared from HCT116 cells that had been transiently transfected with EGFP-PLK4 or -PLK4 K41M in conjunction with control or PLK4 siRNA and western blotted with anti-PLK4 CPB and anti-PLK4 pS305 antibodies. The anti-PLK4 pS305 antibody was found to recognize specifically the active form of the kinase in vivo.

Figure 5 describes an amino acid sequence of PLK4. However, sequences containing polymorphisms and synonyms are included (e.g. Uniprot PLK4_HUMAN O00444)

DETAILED DESCRIPTION OF THE INVENTION

It is demonstrated that PLK4 itself is its own substrate and multiple autophosphorylation sites within the protein are identified. It is shown that autophosphorylation of S305 can be used as a marker of PLK4 activity.

EXPERIMENTAL DATA AND EXAMPLES

Material & Methods

Recombinant DNA Technology, Production of PLK4 Proteins and Site directed mutagenesis

All SUMO fusion plasmids were created by polymerase chain reaction using the full length PLK4 as a template and cloned into a modified version of pSUMO (Invitrogen[®]). Fusion proteins were expressed in BL21 (DE3) cells (Invitrogen[®]) and

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purified with HIS-Select HF Nickel affinity gel (Sigma[®]). Site directed mutagenesis was performed using a Quickchange XL mutagenesis kit (Stratagene[®]) according to manufacturers protocol. Protein expression, purification condition and all primers sequences are further illustrated below.

5

Cells, Transfections and Protein Detection

HCT116 colorectal carcinoma cells were cultured in McCoy's 5A media (Gibco[®]) and Hams F12 media (Gibco[®]) supplemented with 1% L-Glutamine (Gibco[®]), 1% gentamycin and 10% fetal calf serum (Gibco[®]) in a humidified incubator (37°C, 5% CO₂). HeLa centrin-1 GFP cells were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine.

PLK4 plasmids and/or siRNAs were transfected with Lipofectamine 2000 (Invitrogen[®]). PLK4 specific (5'-AAGGACCTTATTCACCAGTTA-3') and Mismatch (Ambion[®], 4611) siRNA were used at 50nM. For myc-tagged and EPFG tagged PLK4 experiments, cells were lysed in triple detergent buffer. For experiments including the detection of phosphorylated S305, cells were lysed in boiling buffer. Lysis buffer components can be found below. Proteins were transferred to nitrocellulose membrane after SDS-PAGE and detected with appropriate antibodies.

20

Antibodies

Primary antibodies were obtained from the following sources: humanized anti-ninein single chain antibody (Bornens laboratory), anti-gamma tubulin monoclonal antibody (Sigma[®]), anti-C-Nap1 (BD Biosciences[®]), anti-PLK4 antibody (Abcam[®]), anti-GAPDH (Abcam[®]). Anti-PLK4 antibodies, detecting the cryptic Polobox (CPB) or phosphorylated S305 (pS305), were produced and affinity purified by Agro-Bio. HRP-secondary antibodies were obtained from Santa Cruz[®]. Alexa488 and Alexa680 secondary antibodies were obtained from Molecular Probes[®]. Cy3, Cy5, AMCA secondary antibodies were obtained from Jackson Laboratories[®].

30

Kinase Reactions, SDS-PAGE, Protein Determination and Radioactive Analysis

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Kinase reactions were incubated for 1 hour at 22°C using the following buffer: 50mM Hepes, 50mM NaCl, 10mM MgCl₂, 1mM NaF, 5μM Xylene cyanole, 1μM ATP, 1mM DTT, 0.3μCi ³³P-γ-ATP and PLK4 recombinant protein. The samples were separated on a 4-12% Bis Tris NuPage gel (Invitrogen[®]), stained with SyproRuby (Molecular Probes[®]) and imaged using a LumiImager (Roche[®]). Gels were destained, dried on Whatman[®] 3MM paper, exposed onto a phosphor-imager screen and imaged using the Typhoon[®]. ImageQuant[®] software was used to quantify the intensity of the bands.

10 *Peptide Synthesis and Phosphorylation Analysis*

A proprietary peptide library containing putative phosphorylation sites in PLK4 was prepared by Jerini[®] using their proprietary microscale technology. The peptides, corresponding locations in PLK4 and mutations of each peptide scanned are displayed in Table I. The peptides were subjected to a kinase reaction using purified SUMO-PLK4₁₋₂₈₅ and processed as described above.

peptide no.	Biotin-Ttds-Glycinamide	derived from:	site	mutation
1	GQRDSSYYWEIE	c-Raf (positive control)		
2	GQRDSAYYWEIE	c-Raf S339A		
3	GQRDASYWEIE	c-Raf S338A		
4	NLLGKG <u>S</u> FAGVYR	PLK4 kin domain	Ser22	
5	QLKHPS <u>I</u> LELYNY	PLK4 kin domain	Ser71	
6	LYNYFED <u>S</u> NYVYL	PLK4 kin domain	Ser82	
7	ILHRDLT <u>L</u> <u>S</u> NLLL	PLK4 kin domain	Thr138 Ser140	
8	ILHRDL <u>A</u> <u>L</u> <u>S</u> NLLL	PLK4 kin domain	Thr138 Ser140	T138A
9	ILHRDLT <u>L</u> <u>A</u> NLLL	PLK4 kin domain	Thr138 Ser140	S140A
10	<u>S</u> AHGLE <u>S</u> DVW <u>S</u> LG	PLK4 kin domain	Ser192	
11	DYEMP <u>S</u> FL <u>S</u> IEAK	PLK4 kin domain	Ser232 Ser235	
12	DYEMP <u>A</u> FL <u>S</u> IEAK	PLK4 kin domain	Ser232 Ser235	S232A

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13	DYEMPS <u>F</u> L <u>A</u> IEAK	PLK4 kin domain	Ser232 Ser235	S235A
14	DYEMPT <u>F</u> L <u>S</u> IEAK	PLK4 kin domain	Ser232 Ser235	S235T *
15	LRRNPADRL <u>S</u> L <u>S</u> S	PLK4 kin domain	Ser255	
16	LRRNPADRL <u>A</u> L <u>S</u> S	PLK4 kin domain	Ser255	S255A
17	DRL <u>S</u> L <u>S</u> S <u>V</u> LDHPF	PLK4 kin domain	Ser257-258	
18	DRL <u>S</u> L <u>A</u> <u>S</u> <u>V</u> LDHPF	PLK4 kin domain	Ser257-258	S257A
19	DRL <u>S</u> L <u>S</u> <u>A</u> <u>V</u> LDHPF	PLK4 kin domain	Ser257-258	S258A
20	<u>S</u> I <u>S</u> G <u>S</u> LFDKRRL	PEST sequence 1	Ser303 Ser305	
21	<u>S</u> I <u>S</u> G <u>A</u> LFDKRRL	PEST sequence 1	Ser303 Ser305	S305A
22	<u>S</u> I <u>A</u> G <u>S</u> LFDKRRL	PEST sequence 1	Ser303 Ser305	S303A
23	PLPNKMT <u>V</u> FPKNK	after destruction box	Thr323	
24	DGNS <u>F</u> Y <u>T</u> QWGNQ	Linker region	Ser342 T345	
25	DGN <u>A</u> FY <u>T</u> QWGNQ	Linker region	Ser342 T345	S342A
26	DGNS <u>F</u> Y <u>A</u> QWGNQ	Linker region	Ser342 T345	T345A
27	NEERY <u>S</u> P <u>T</u> DNNAN	Linker region	Ser421	
28	NEERY <u>A</u> P <u>T</u> DNNAN	Linker region	Ser421	S421A
29	SSSG <u>S</u> FERPDNNQ	Linker region	Ser443	
30	SSSG <u>A</u> FERPDNNQ	Linker region	Ser443	S443A
31	<u>A</u> <u>A</u> <u>A</u> G <u>S</u> FERPDNNQ	Linker region	Ser443	S439-441A
32	EYDS <u>I</u> SPNRDFQG	Linker region	Ser497 Ser499	
33	EYD <u>A</u> ISPNRDFQG	Linker region	Ser497 Ser499	Ser497A
34	EYD <u>S</u> I <u>A</u> PNRDFQG	Linker region	Ser497 Ser499	Ser499A
35	<u>S</u> DGNT <u>T</u> ITIIYPNG	Crypto-Polo Box	Thr 645 647	
36	<u>S</u> DGN <u>A</u> ITIIYPNG	Crypto-Polo Box	Thr 645 647	T645A
37	<u>S</u> DGNT <u>I</u> <u>A</u> IYYPNG	Crypto-Polo Box	Thr 645 647	T647A
38	DNIS <u>R</u> Y <u>S</u> FDNLPE	Crypto-Polo Box	Ser671 674	
39	DNIS <u>R</u> Y <u>A</u> FDNLPE	Crypto-Polo Box	Ser671 674	S674A
40	DN <u>I</u> <u>A</u> RY <u>S</u> FDNLPE	Crypto-Polo Box	Ser671 674	S671A
41	K <u>S</u> PK <u>I</u> Y <u>F</u> TRYAK	Crypto-Polo Box	Thr 704 707	
42	K <u>S</u> PK <u>I</u> <u>A</u> Y <u>F</u> TRYAK	Crypto-Polo Box	Thr 704 707	T704A
43	K <u>S</u> PK <u>I</u> Y <u>F</u> <u>A</u> RYAK	Crypto-Polo Box	Thr 704 707	T707A

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44	KIHK <u>T</u> EDFIQVIE	Crypto-Polo Box	Thr736	
45	QVIEK <u>T</u> GK <u>S</u> Y <u>T</u> LK	Crypto-Polo Box	Ser749	
46	QVIEK <u>A</u> GK <u>S</u> Y <u>T</u> LK	Crypto-Polo Box	Ser749	T746A
47	QVIEK <u>T</u> GK <u>A</u> Y <u>T</u> LK	Crypto-Polo Box	Ser749	S749A
48	QVIEK <u>T</u> GK <u>S</u> Y <u>A</u> LK	Crypto-Polo Box	Ser749	T751A
49	PP <u>S</u> VD <u>S</u> NY <u>P</u> TRDR	Crypto-Polo Box	Ser824	
50	PP <u>S</u> VD <u>A</u> NY <u>P</u> TRDR	Crypto-Polo Box	Ser824	S824A
51	PP <u>S</u> VD <u>S</u> NY <u>P</u> <u>A</u> RDR	Crypto-Polo Box	Ser824	T828A
52	RDRA <u>S</u> FNRMVMH	Crypto-Polo Box	Ser833	
53	G <u>T</u> DI <u>S</u> NSL <u>K</u> DC	Crypto-Polo Box	Ser873 874	
54	G <u>T</u> DI <u>A</u> NSL <u>K</u> DC	Crypto-Polo Box	Ser873 874	S873A
55	G <u>T</u> DI <u>S</u> <u>A</u> NSL <u>K</u> DC	Crypto-Polo Box	Ser873 874	S874A
56	G <u>T</u> DI <u>S</u> <u>A</u> <u>N</u> AL <u>K</u> DC	Crypto-Polo Box	Ser873 874	S874A S876A
57	AQLLK <u>S</u> VFVKNVG	PEST sequence 3	Ser890	
58	VGWAT <u>Q</u> LT <u>S</u> GAVW	PEST seq 3 + Polo Box	Thr899 Ser904	
59	VGWAA <u>Q</u> LT <u>S</u> GAVW	PEST seq 3 + Polo Box	Thr899 Ser904	T899A
60	VGWAT <u>Q</u> LA <u>S</u> GAVW	PEST seq 3 + Polo Box	Thr899 Ser904	T903A
61	VGWAT <u>Q</u> LT <u>A</u> GAVW	PEST seq 3 + Polo Box	Thr899 Ser904	S904A
62	VQFNDG <u>S</u> QLVVQA	PEST seq 3 + Polo Box	Ser915	
63	GV <u>S</u> SI <u>S</u> YT <u>S</u> PNGQ	Polo Box	Ser927	
64	GV <u>S</u> SI <u>A</u> YT <u>S</u> PNGQ	Polo Box	Ser927	S927A
65	GV <u>A</u> SI <u>S</u> YT <u>S</u> PNGQ	Polo Box	Ser927	S924A
66	GV <u>S</u> AI <u>S</u> YT <u>S</u> PNGQ	Polo Box	Ser927	S925A
67	QKLQCL <u>S</u> SILLMF	Polo Box	Ser956-Ser957	
68	QKLQCL <u>A</u> SILLMF	Polo Box	Ser956-Ser957	S956A
69	QKLQCL <u>S</u> <u>A</u> ILLMF	Polo Box	Ser956-Ser957	S957A
70	GQRDSSYYWEIE	c-Raf (positive control)	Raf duplo	
71	RGQRDSSYYWE	c-Raf (positive control)	shorter	
72	RGQRDA <u>S</u> YYWE	c-Raf S339A	shorter	S339A
73	RGQRDS <u>A</u> YYWE	c-Raf S338A	shorter	S338A

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*: polymorphism

single underlined: putative site on PLK4

Double underline + italics: mutation

5

Immunofluorescence imaging

HeLa centrin-1 GFP cells growing on fibronectin/collagen-coated coverslips were washed once with PBS and fixed with methanol at -20°C for 20 minutes. Coverslips were sequentially washed with PBS and antibody blocking buffer: PBS containing 1% bovine serum albumin fraction V (Sigma[®]) and 0.5% Triton X-100 (Sigma[®]) prior to adding primary antibodies diluted in antibody blocking buffer. Cells were stained with primary antibodies for 1 hour at ambient temperature and washed extensively with antibody blocking buffer before incubating with secondary antibodies, diluted in the same buffer, for 30 minutes at ambient temperature. The coverslips were washed with antibody blocking buffer, DNA stained with a 0.2 µg/ml solution of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma[®]), and sequentially washed with antibody dilution buffer and distilled water. After allowing to air-dry, the coverslips were mounted onto glass slides using Mowiol mounting medium. Images were captured on a Leica DMRA2 microscope, fitted with a CoolSNAP camera (Princeton Instruments[®]), using a 100X 1.4 N.A. objective lens (Leica[®]) and Metamorph software (Universal Imaging[®]). Processing of images was carried out using Metamorph software.

Recombinant DNA Technology, Production of PLK4 Proteins and Site directed mutagenesis

BL21 (DE3) competent cells (Invitrogen[®]) were transformed with the recombinant DNA according to manufacturer's protocol. Following culture upon agar plates containing Kanamycin and 1% glucose, a single colony was resuspended in 2YT media containing Kanamycin and 0.1% glucose for 3 hours. 1ml of the pre-culture was then incubated in 400ml auto-induction medium (Novagen[®]) without glucose at 37° for 3 hours to reach an OD (600nm) of 0.6. At this point the temperature was reduced to

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23°C for another 16 hours. The samples were spun down at 3500rpm for 25 minutes at 4°C. The pellets were then resuspended in 10ml ice cold MilliQ and 10ml 2x lysis buffer containing 100mM Tris pH8, 600mM NaCl, 0.015% β mercapto-ethanol, 2% NP40, 40% glycerol and protease inhibitors (Complete[®], Roche[®]). A sonication for 5 seconds, 12 times at low output was performed to complete lysis. The proteins, recuperated after centrifugation at 25000 rpm for 20 minutes at 4°C, were bound to HIS-Select HF Nickel affinity gel (Sigma[®]) for 2.5 hours under agitating conditions at 4°C. The HIS beads were spun down at 5000 rpm for 5 minutes, washed twice with 20mM Tris pH8, 300mM NaCl, 10mM Imidazole pH8, 0.2% NP40, 20% glycerol and 0.007% β mercapto-ethanol for 10minutes under agitating conditions at 4°C. Finally the proteins were eluted from the beads with 20mM Tris pH8, 300mM NaCl, 500mM Imidazole pH8, 0.2% NP40, 20% glycerol and 0.007% β mercapto-ethanol for 15minutes under agitating conditions at 4°C and collecting the supernatant after centrifugation at 5000rpm for 5minutes at 4°C.

15

The primers used for sited directed mutagenesis were

S305A FW: 5'-CCAGTACCAGTATAAGTGGTGC GTTATTTGACAAAAGAAGAC-3' ;

S305A RV: 5'-GTCTTCTTTTGTC AAATAACGCACCACTTATACTGGTACTGG-3' ;

20 S305D FW: 5'-CCAGTACCAGTATAAGTGGTGATTATTTGACAAAAGAAGAC-3'

S305D RV: 5'-GTCTTCTTTTGTC AAATAAATCACCACCTTATACTGGTACTGG-3'.

Cells, Transfections and Protein Detection

25 For myc-tagged and EPFG-tagged PLK4 experiments, cells were lysed in triple detergent buffer: 50mM Tris pH8, 150mM NaCl, 0.1% SDS, 1% NP-40, 0.5% Na-DOC, protease and phosphatase inhibitor cocktail (Roche[®]).

For experiments including the detection of phosphorylated S305, cells were lysed in boiling buffer: 1% SDS, 100mM Tris pH7.4, 1mM Na₃VO₄.

30

Results

Detection of an autophosphorylation in the amino terminal region of PLK4

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- PLK4 contains an amino terminal kinase domain, a carboxy terminal crypto Polobox and Polobox domain and three PEST regions (Figure 1A). When purified full length recombinant PLK4 from *E. coli* was subjected to an *in vitro* kinase reaction, autophosphorylation was observed (Figure 1A). Multiple bands were observed indicating that the full length protein had been heavily degraded, but also raised the possibility that phosphorylation occurred at several sites within the protein. To investigate this further we generated a series of PLK4 fragments to determine where autophosphorylation primarily occurred.
- 10 A PLK4 truncation, corresponding to residues 1 to 597, lacking the CPB and the Polobox domains was generated in fusion with SUMO (Figure 1B). Deletion of the carboxy terminal region is believed to render the protein more stable, most likely due to the fact that only one of the three PEST sequences is present in the amino terminal end. This SUMO-PLK4₁₋₅₉₇ fusion protein was subjected to an *in vitro* kinase reaction and
- 15 was found to be autophosphorylated as well. Coomassie staining showed that some protein degradation still occurred, with a large proportion of the degradation products being shorter N-terminal fragments of PLK4. This was confirmed by an identical kinase reaction being western blotted using an antibody targeting the amino terminal region of PLK4.
- 20 The phosphorylation detected in the truncated PLK4₁₋₅₉₇ protein could either be due to inter- or intra autophosphorylation. To investigate this, we generated a kinase dead version of PLK4₁₋₃₆₇ K41M, where lysine41 within the ATP binding pocket, had been mutated to methionine. An *in vitro* kinase assay demonstrated that the kinase was inactive (Figure 1C). We then used PLK4₁₋₃₆₇ K41M as a substrate in an *in vitro* kinase
- 25 reaction with active PLK4₁₋₂₈₅, corresponding to the kinase domain of the enzyme. Interestingly, phosphorylation of PLK4₁₋₃₆₇ K41M was observed, along side that of the kinase domain, indicating that inter molecular autophosphorylation had occurred. In brief, these results showed that autophosphorylation sites are present the amino terminus of PLK4. We then decided to investigate if other autophosphorylation sites
- 30 existed within the protein.

Inter-molecular phosphorylation of PLK4 detectable in various regions of the protein

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To determine if autophosphorylation occurred elsewhere in the protein, a series of PLK4 fragments fused with SUMO were created (Figure 2A). All of the fragments lacked kinase activity and thus could be used as substrates for phosphorylation by PLK4₁₋₂₈₅. A significant phosphorylation was observed in all fragments apart from
5 PLK4₈₀₃₋₈₈₉ and PLK4₈₈₈₋₉₇₀ where weak and no phosphorylation was observed, respectively. PLK4₃₆₅₋₆₃₉ had a similar migration distance as PLK4₁₋₂₈₅, therefore masking the phosphorylation signal. SyproRuby staining confirmed that similar molarities of substrate protein were used in the kinase reaction (Figure 2B). These results indicated that multiple sites at various regions of the protein can be
10 phosphorylated by PLK4.

S305 is a major autophosphorylation site in PLK4

Commercially available peptide libraries were used to screen for peptidic substrates of
15 PLK4₁₋₂₈₅. From these results a consensus PLK4 phosphorylation sequence was derived (data not shown). It was further refined using bioinformatics modeling tools and confirmed on proprietary peptide libraries (Bonnet *et al.* – manuscript in preparation). This algorithm was used to recognize sequences on PLK4 that corresponded to the consensus sequence and identify potential phosphorylation sites (Figure 3A). The
20 corresponding 13-mer peptides were synthesized and used to evaluate which residues were phosphorylated the strongest. In the case where multiple serines or threonines were present on the same peptide, mutant peptides with alanine substitutions were also examined in order to unambiguously determine the phosphorylation site (see Table I for a complete list of scanned peptides). Four peptides contained serine/threonine
25 residues that were phosphorylated by PLK4₁₋₂₈₅ (Figure 3B). The strongest phosphorylation was observed on peptide 20 containing S305. This peptide contains another serine at residue 303, however, when this was mutated to alanine the phosphorylation of the peptide still occurred (peptide 22). Concurringly, when the serine at residue 305 was mutated to alanine, the phosphorylation was abrogated
30 (peptide 21). This confirmed that the phosphorylation on S305 was the key phosphorylation site in this peptide. Another serine, residue 342, which fitted well to the consensus sequence, was found to be poorly phosphorylated by PLK4₁₋₂₈₅. The second strongest autophosphorylation was observed on peptide 7, which contains

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threonine 138 and serine 140. It was, however, impossible to attribute the phosphorylation to either of these residues definitively as mutations to alanine did not result in the disappearance of phosphorylation. This indicates that the two sites can independently from one another produce a strong phosphorylation signal. Weak phosphorylation of peptide 67 was also observed, which contained serine residues 956 and 957. Neither of the two serines is more important than the other as phosphorylation of serine to alanine mutants was still observed. Residues 956 and 957 are located at the end of the PoloBox and may perhaps play a role in protein binding. As S305 appeared the major phosphorylation site on PLK4, a phospho-specific antibody was raised against this epitope to enable a more detailed investigation of this phosphorylation site. To detect S305 phosphorylation *in vitro*, affinity purified rabbit antibodies against the phosphorylated S305 site were used to probe PLK4₁₋₃₆₇, PLK4₁₋₃₆₇ K41M and lambda phosphatase treated PLK4₁₋₃₆₇ after kinase reactions. Autoradiography demonstrated that the PLK4₁₋₃₆₇ was autophosphorylated (Figure 4A-left panel) whereas no signal was observed in PLK4₁₋₃₆₇ K41M and lambda phosphatase treated PLK4₁₋₃₆₇. The amount of protein for each sample was comparable as determined by SyproRuby staining (Figure 4A-middle panel). When subjected to western blotting and probed with the phospho-specific S305 antibody (pS305), the phosphorylation of the PLK4₁₋₃₆₇ could clearly be detected (Figure 4A-right panel). No signal was observed from PLK4₁₋₃₆₇ K41M or when the PLK4₁₋₃₆₇ was treated with lambda phosphatase. We further asked if the autophosphorylation of PLK4 was detectable *in vivo* as well.

The colorectal carcinoma cell line, HCT116, was transiently transfected with plasmids encoding EGFP fused with either PLK4 or PLK4 K41M. Western blotting with antibodies against the CPB domain or pS305 of PLK4 was carried out (Figure 4B). PLK4 and PLK4 K41M were detected with the CPB antibody, however only active PLK4 was detected with the pS305 antibody indicating that this residue is exclusively phosphorylated by PLK4 in cells. We also observed the disappearance of the pS305 signal when the antibody was pre-incubated with the peptide used to raise the antibody (data not shown). Upon co-transfection of a PLK4 specific siRNA, complete ablation of total PLK4 and PLK4 K41M was observed and, more importantly, resulted a loss of signal upon detection with the pS305 antibody. Additionally, when PLK4 was immunoprecipitated with the pS305 antibody from HCT116 cells lysates, we were only able to detect PLK4 from cells overexpressing the active form of the kinase (data not

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shown). These data confirm that the pS305 antibody specifically recognizes its phosphorylated epitope and that S305 is an autophosphorylation site rather than a site for another kinase. This shows that S305 is a genuine autophosphorylation site in PLK4 as it could be detected both *in vitro* and *in vivo*.

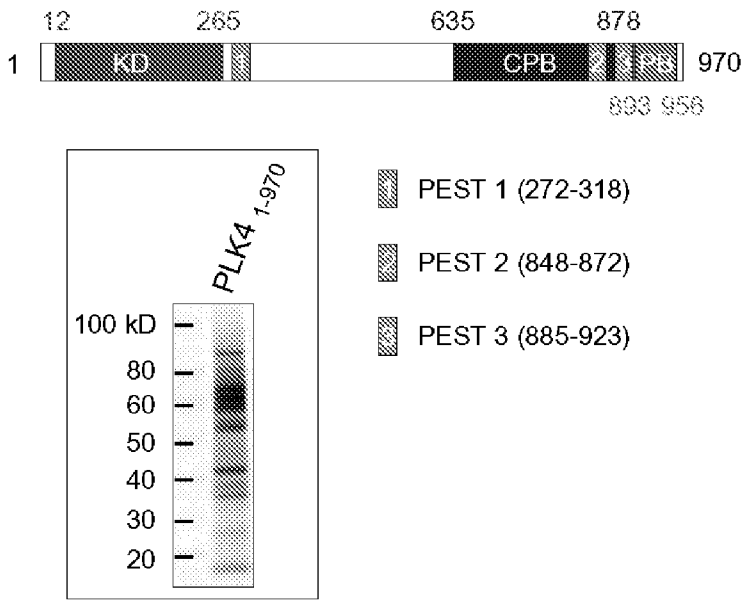
-18-

Claims

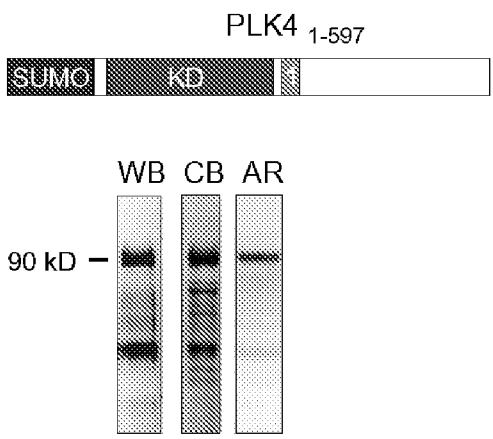
1. A method for determining the activity of PLK4 comprising the steps of:
 - providing a sample to be tested for PLK4 activity,
 - determining the level of autophosphorylation of S305 of PLK4 in said sample, and
 - determining the activity of PLK4 activity in the sample of cells, wherein the level of S305 autophosphorylation directly correlates with the level of PLK4 activity.
2. The method as in claim 1, wherein the level of autophosphorylation of S305 of PLK4 is determined using an immunostaining procedure with an antibody reagent that is specific to the S305 autophosphorylation site on PLK4
3. The method according to claim wherein the antibody reagent is fluorescently or radioactively labeled.
4. The method according to claim 1, wherein the level of autophosphorylation of S305 of PLK4 is determined using a sandwich ELISA assay in which a first antibody reagent is specific to PLK4 protein and a second antibody reagent is specific to the S305 autophosphorylation sites on PLK4.

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A



B



C

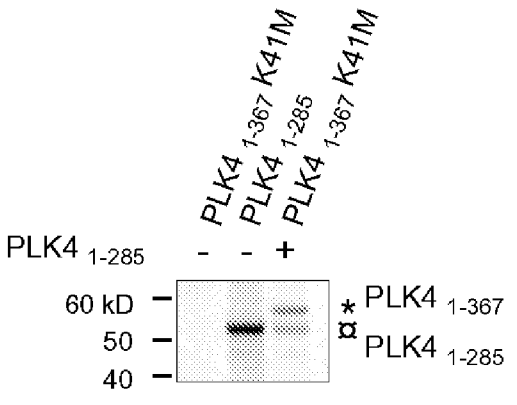
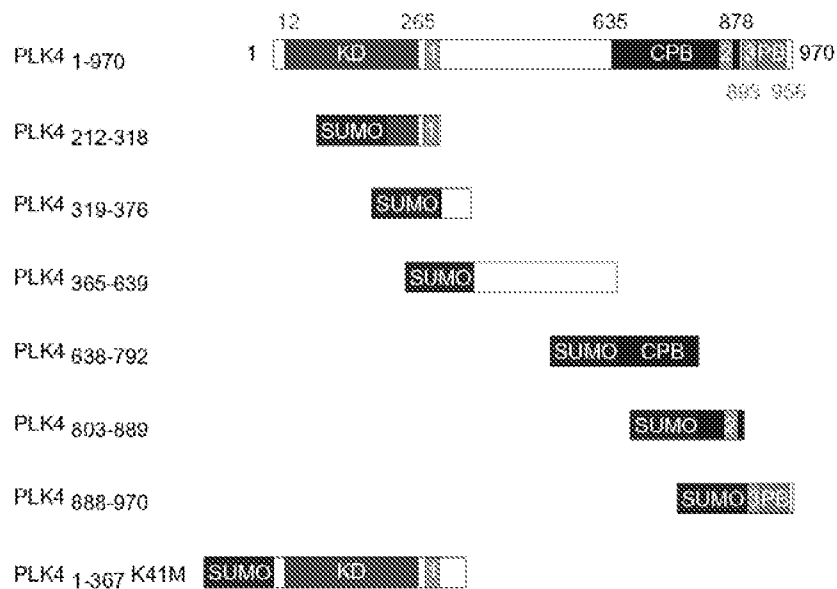


Fig 1

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A



B

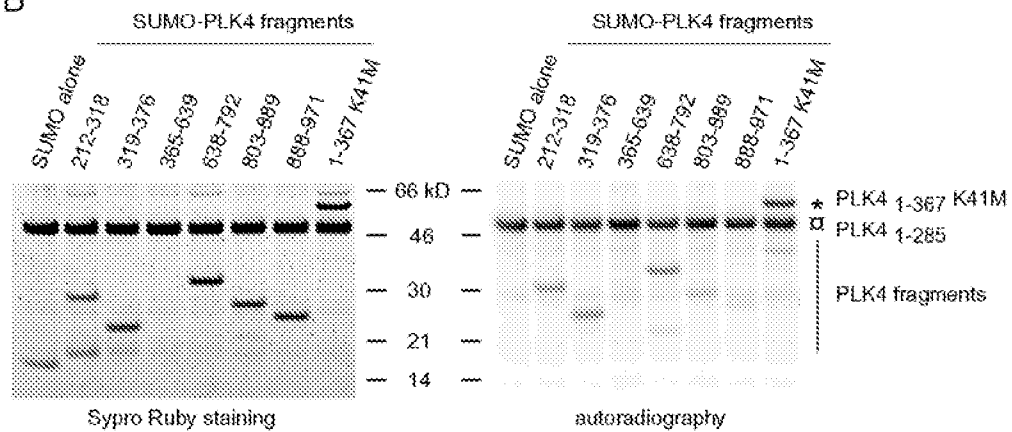


Fig 2

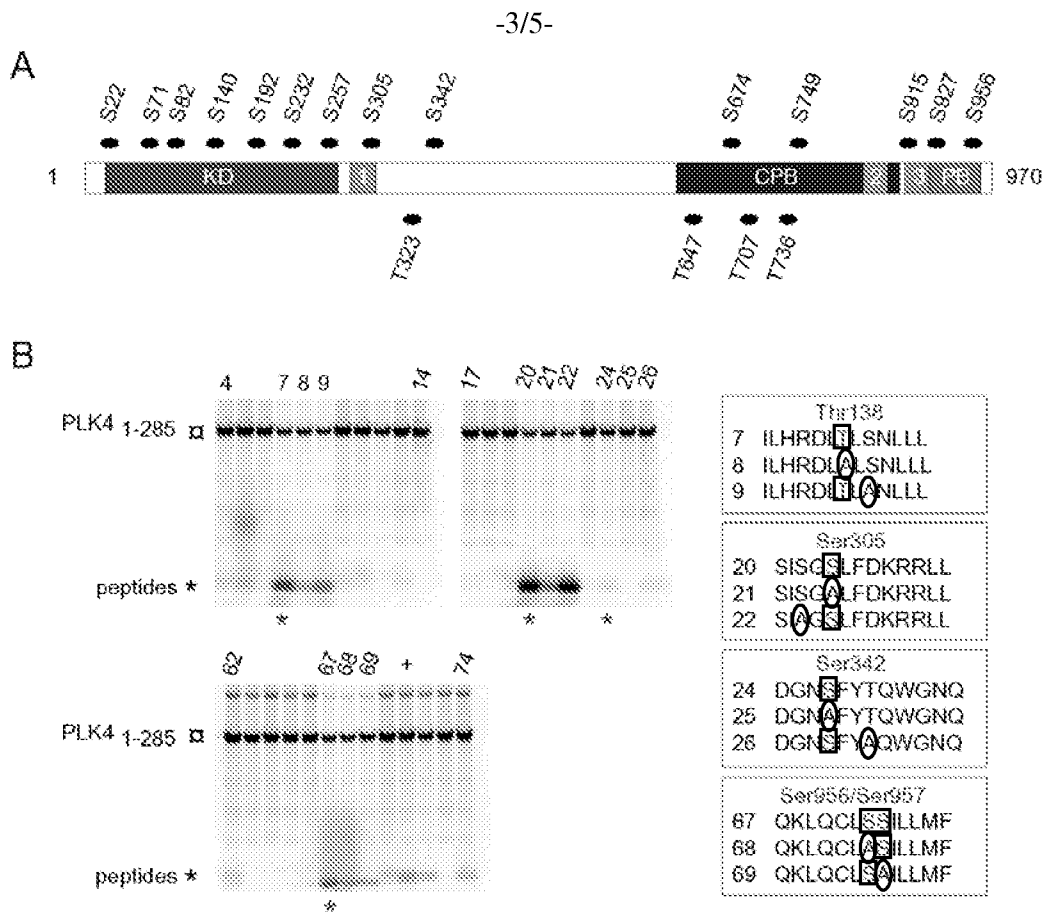


Fig. 3

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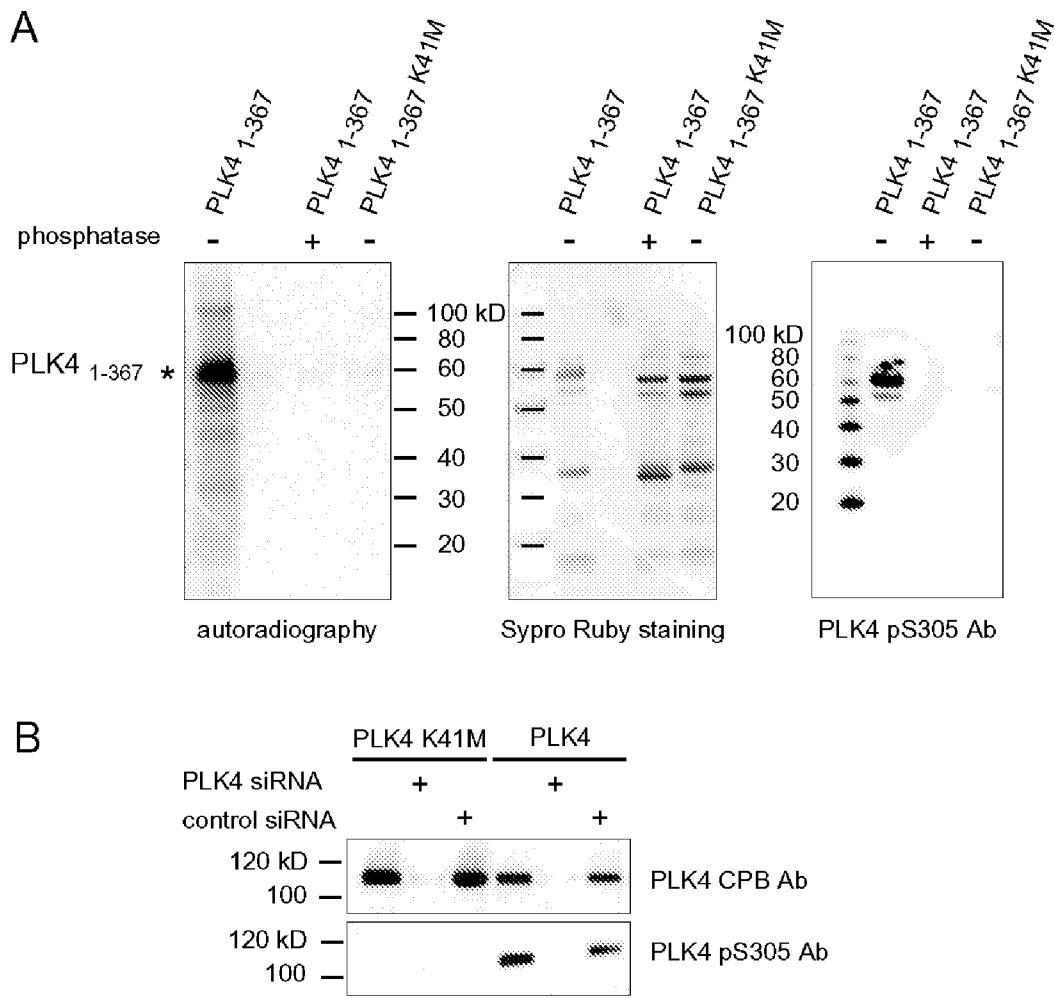


Fig. 4

-5/5-

10	20	30	40	50	60
MATCIGEKIE	DFKVGNLLGK	GSFAGVYRAE	SIHTGLEVAI	KMIDKKAMYK	AGMVQRVQNE
70	80	90	100	110	120
VKIHQQLKHP	SILELYNYFE	DSNYVYLVLE	MCHNGEMNRY	LKNRVKPFSE	NEARHFMHQI
130	140	150	160	170	180
ITGMLYLHSH	GILHRDLTSL	NLLLTRNMNI	KIADFGLATQ	LKMPHEKHHT	LCGTPNYISP
190	200	210	220	230	240
EIATRSAGHL	ESDVWSLGCM	FYTLLIGRPP	FDTDTVKNTL	NKVVLADYEM	PSFLSIEAKD
250	260	270	280	290	300
LIHQLLRRNP	ADRLSLSSVL	DHPFMSRNSS	TKSKDLGTVE	DSIDSGHATI	STAITASSST
310	320	330	340	350	360
SISGSLFDKR	RLLIQQLPN	KMTVFPKNKS	STDFSSSGDG	NSFYTQWGNQ	ETSNSGRGRV
370	380	390	400	410	420
IQDAEERPHS	RYLRRAYSSD	RSGETSNSQSQ	AKTYTMERCH	SAEMLSVSKR	SGGGENEERY
430	440	450	460	470	480
SPTDNNANIF	NFFKEKTSSS	SGSFERPDNN	QALSNHLCPG	KTPFPFADPT	PQTETVQQWF
490	500	510	520	530	540
GNLQINAHLR	KTTEYDSISP	NRDFQGHFDL	QKDTSKNAWT	DTKVKKNSDA	SDNAHSVKKQ
550	560	570	580	590	600
NTMKYMTALH	SKPEIIQDEC	VFGSDPLSEQ	SKTRGMEPPW	GYQNRTLRSI	TSPLVAHRLK
610	620	630	640	650	660
PIRQKTKKAV	VSILDSEEVV	VELVKEYASQ	EYVKEVLQIS	SDGNTITIYY	PNGGRGFPLA
670	680	690	700	710	720
DRPPSPTDNI	SRYSFDNLPE	KYWRKYQYAS	RFVQLVRSKS	PKITYFTRYA	KCILMENSPG
730	740	750	760	770	780
ADFEVWFYDG	VKIHKTEDFI	QVIEKTGKSY	TLKSESEVNS	LKEEIKMYMD	HANEHGRICL
790	800	810	820	830	840
ALESIISEEE	RKTRSAPFFP	IIIGRKPGST	SSPKALSPPP	SVDSNYPTRE	RASFNRMMVMH
850	860	870	880	890	900
SAASPTQAPI	LNPSMVTNEG	LGLTTTASGT	DISSNSLKDC	LPKSAQLLKS	VFVKNVGVAT
910	920	930	940	950	960
QLTSGAVVWQ	FNDGSQLVVQ	AGVSSISYTS	PNGQTTRYGE	NEKLPDYIKQ	KLQCLSSILL
970					
MFSNPTPNFH					

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/050179

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/543 C12N9/12 G01N33/53 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEUNG ET AL: "Determination of the Plk4/Sak consensus phosphorylation motif using peptide spots arrays" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 581, no. 1, 23 December 2006 (2006-12-23), pages 77-83, XP005815647 ISSN: 0014-5793 *the whole document, especially the abstract and page 82, right-hand column* ----- -/-	1-4

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

5 March 2009

Date of mailing of the international search report

13/03/2009

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/050179

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JANG, Y-J., ET AL: "Phosphorylation of Threonine 210 and the Role of Serine 137 in the regulation of Mammalian Polo-like Kinase" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 46, 15 November 2002 (2002-11-15), pages 44115-44120, XP002483097 *the whole document, especially the abstract, page 44115, right-hand column, lines 33-42 and page 44119, right-hand column, line 1 - page 44120, left-hand column, line 2*	1-4
A	WO 2007/146436 A (VERTEX PHARMA [US]; BROWN KIERON [GB]; CHEETHAM GRAHAM MT [GB]; RENWIC) 21 December 2007 (2007-12-21) *abstract, paragraphs [173]-[176]*	1-4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/050179

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007146436 A	21-12-2007	US 2008293579 A1	27-11-2008